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Proteins containing a Caspase-Associated Recruitment Domain (CARD) have previously been shown to serve as key regulators of tumor cell survival as well as regulators of other cellular processes, such as cytokine production. Interleukin-1 beta (IL-1β) is a pro-inflammatory cytokine which has been found to be associated with more aggressive and invasive breast tumors. Previous work (year 1) focused on the initial cloning and functional characterization of NAC-X (now named CLAN), a novel protein containing CARD, NACHT and LRR domains. NAC-X was previously found to regulate caspase-1 and IL-1β activation, more recent experiments have determined that NAC-X may also affect the functions of other NACHT-containing proteins via heterotypic associations through its NACHT domain. Futhermore, NAC-X was found to elicit an inflammatory response in macrophages following exposure to the bacterial component LPS, suggesting a role for this gene in the innate immune system. It remains to be determined whether NAC-X-mediated IL-1β secretion may function as a growth or survival factor in breast tumors.

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Introduction

Proteins containing a Caspase-Associated Recruitment Domain (CARD) have previously been shown to serve as key regulators of tumor cell survival as well as regulators of other cellular processes, such as cytokine production. Interleukin-1 beta (IL-1β) is a pro-inflammatory cytokine which has been found to be associated with more aggressive and invasive breast tumors (Jin, Yuan et al. 1997), (Kurtzman, Anderson et al. 1999). Previous work (year 1) focused on the initial cloning and functional characterization of NAC-X (now named CLAN), a novel protein containing CARD, NACHT and LRR domains. NAC-X was previously found to regulate caspase-1 and IL-1β activation, more recent experiments have determined that NAC-X may also affect the functions of other NACHT-containing proteins via heterotypic associations through its NACHT domain. Futhermore, NAC-X was found to elicit an inflammatory response in macrophages following exposure to the bacterial component LPS, suggesting a role for this gene in the innate immune system. It remains to be determined whether NAC-X-mediated IL-1β secretion may function as a growth or survival factor in breast tumors.

Body

Specific Aim 1:

Specific Aim 1 of this research project was to determine the expression pattern of NAC-X in normal and malignant mammary tissues as well as in normal human tissues. Tasks 1 and 2 were completed during the first year of the award (screening of NAC-X expression and cloning of full-length NAC-X, respsectively). The goal of Task 3 was to genereate a polyclonal antibody against NAC-X for use in immunoblotting or immunohistochemistry. The first peptide used for immunization failed to produce a viable NAC-X antibody. A second attempt using a different peptide resulted in an antibody which is capable of detecting over-expressed, but not endogenous, NAC-X using western blotting (figure 1).

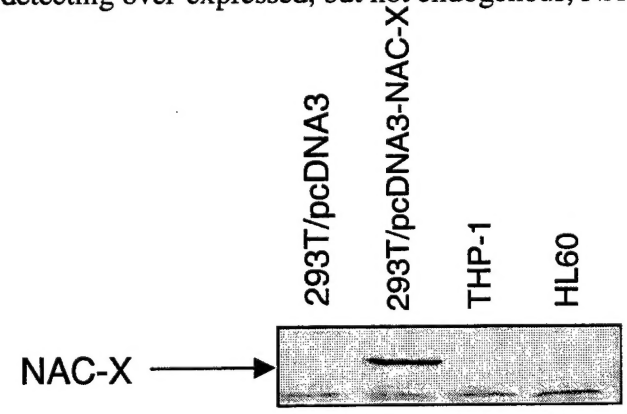

Figure 1. Detection of NAC-X using rabbit polyclonal antibody. Anti-NAC-X antibody is capable of detecting over-expressed NAC-X (in 293T Lysates) but not endogenous NAC-X in THP-1 or HL60 lysates (both NAC-X positive by RT-PCR analysis).

The low detection threshold may be due to the scarcity of endogenous NAC-X within the cell. Attempts to use this antibody to immunoprecipitate NAC-X are ongoing.

Specific Aim 2:

The goal of Specific Aim 2 was to evaluate the associations of NAC-X with other CARD-containing proteins involved in apoptosis. This aim was completed in year 1, leading to the discovery that NAC-X associated with caspase-1, Nod1, Nod2, and NAC. NAC-X was also found to oligomerize with itself through its NACHT (nucleotide-binding) domain. In an effort to expand on this specific aim, the ability of the NACHT domain of NAC-X to mediate binding to other NACHT family proteins was investigated. The NACHT domain of NAC-X was found to hetero-oligomerize with the NACHT domains of Nod2, NAC, and crypoyrin (as well as other proteins within this family, data not shown) but not to several proteins lacking a NACHT domain (figure 2).

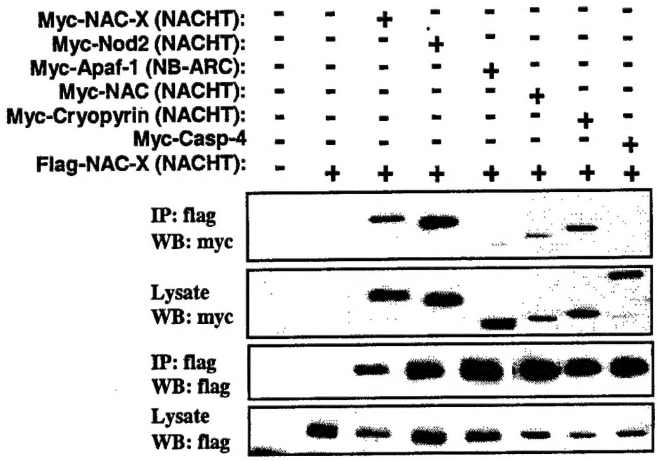

Figure 2. The NACHT domain of NAC-X associates with the NACHT domains from other family members. Expression constructs encoding various NACHT domains or genes lacking NACHT domains (Apaf-1-NB-ARC and Caspase-4) were transiently transfected into 293T cells. Cells were lysed and subjected to immunoprecipitation with anti-myc agarose. Proteins were separated by SDS-PAGE and subjected to immunoblotting with anti-flag or anti-myc antibodies.

Specific Aim 3:

Specific Aim 3 was to determine the specific step(s) in apoptosis pathways regulated by NAC-X. Work in year one determined that NAC-X does not significantly affect BAX- or fas-mediated cell death but does enhance caspase-1-mediated apoptosis. Another pathway by which cells regulate gene expression and survival is through the activation of the NF-κB transcription complex. Since the Nod proteins have been shown to be inducers of NF-κB, (Inohara, Koseki et al. 1999), (Ogura, Inohara et al. 2001), we next investigated the potential effects of NAC-X on this signaling pathway. It was found that NAC-X was capable of inhibiting Nod1- and Nod2-mediated NF-kB activation in a dose-dependent manner (figure 3). Since NAC-X and Nod2 are both expressed in monocytes, these

proteins may actually play more of a role in the inflammatory process than in apoptosis, as the more recent literature would suggest (Inohara, Ogura et al. 2001).

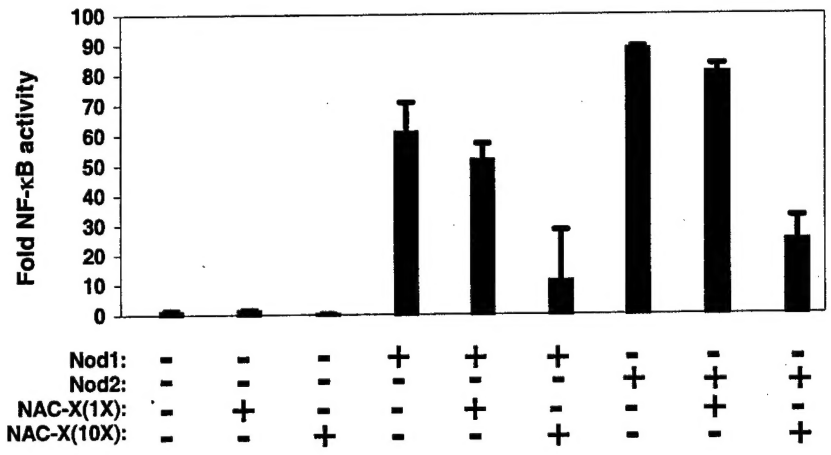

Figure 3. NAC-X inhibits NF-κB activation induced by Nod1 and Nod2. HEK293T cells were seeded into 24 well-plates and transfected the following day with 100 ng of p-NF-κB-luc and 50 ng of pTK-RL reporter gene plasmids together with 100 ng plasmids encoding Nod1 or Nod2 and either 100 ng or 1 μg of plasmid encoding NAC-X. Cells were lysed 24 hr later and luciferase activity was determined using an automated luminometer. Data represent fold induction of NF-κB activity relative to control-transfected cells (mean ± standard deviation [n=3]) after normalization for transfection efficiency based on Renilla luciferase activity.

Specific Aim 4:

The goal of Specific Aim 4 was to analyze the importance of the NAC-X gene for cell survival/cell death *in vivo*. Since recent data has pointed to a role for CLAN in the activation of caspase-1/interleukin-1β, as opposed to its direct involvement in apoptosis, a monocytic cell line over-expressing the full-length NAC-X gene was generated. To accomplish this, a retrovirus was utilized to introduce epitope-tagged NAC-X into THP-1 cells which were subsequently selected for stable expression using G418. Western blotting confirmed the presence of myc-tagged NAC-X protein in cell lysates (data not shown). To evaluate the effects of NAC-X on the function on monocytes/macrophages, the secretion of endogenous interleukin-1β (IL-1β) was examined by ELISA following treatment with bacterial lipopolysaccharide (LPS), a known inducer of this cytokine in macrphages. Figure 4 shows that cells over-expressing NAC-X demonstrate a hyper-response to LPS when compared to control cells (most likely due to increased NAC-X-mediated activation of caspase-1, the protease responsible for cleaving pro-IL-1β into its mature, secreted form).

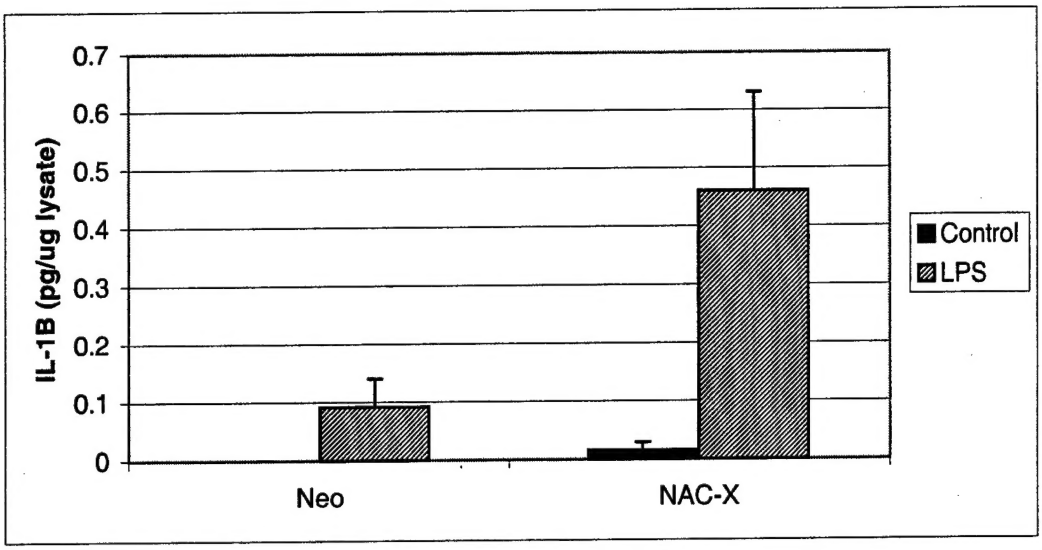

Figure 4. NAC-X mediates the release of IL-1β in monocytic cells following LPS exposure. Stable THP-1 cell lines expressing NAC-X or neomycin resistant control cells (Neo) were differentiated overnight with TPA. 24 hr later, cells were exposed to bacterial LPS (500ng/ml) for 6 hr and supernatants were collected. The presence of IL-1b was detected using and ELISA kit and samples were normalized using total cell lysate protein levels. Data represent mean ± standard deviation [n=3].

Key Research Accomplishments

- A polyclonal antibody capable of recognizing over-expressed (and potentially immunoprecipitated endogenous) NAC-X was successfully generated.
- NAC-X was found to associate with other NACHT family members via its NACHT domain and to affect their ability to activate NF-κB.
- THP-1 cells over-expressing NAC-X secrete higher levels of IL-1β following LPS exposure, demonstrating a role for this gene in controlling cytokine levels.

Reportable Outcomes

Jason S. Damiano, Christian Stehlik, Frederick Pio, Adam Godzik, and John C. Reed. Cloning and Characterizaion of CLAN, a novel CED-4 homolog which regulates caspase-1 activity. Dept. Of Defense Breast Cancer Research Program Meeting, 2002.

Conclusions

During the first year of research, NAC-X was successfully cloned and found to be expressed in a number of human tissues, including breast cancer cell lines. Due to its homology with the cell death regulator Apaf-1, NAC-X was initially believed to play a role in determining apoptotic susceptibility in human cells. However, subsequent experiments

determined that it was actually a regulator of caspase-1, a caspase not usually involved in apoptosis. This caspase is known to control the cleavage and secretion of pro-IL-1β, a pro-inflammatory cytokine that is key to the innate immune response and possibly associated with a more aggressive breast cancer phenotype. Work done during the previous year lead to the successful generation of a polyclonal antibody against NAC-X which still has to be further characterized. Other studies found that NAC-X may affect other similarly structured proteins (of the NACHT family) through interactions mediated by its NACHT domain. Finally, experiments utilizing stable monocytic cell lines demonstrated that NAC-X is capable of mediating cytokine release following stimulation with bacterial components such as LPS. Further studies utilizing NAC-X -over-expressing breast cancer cell lines will determine whether or not NAC-X is a mediator of IL-1β secretion in breast tumors. In conclusion, NAC-X is a regulator of the pro-inflammatory cytokine IL-1β, which is known to be an important element of the innate immune response and possibly a survival/growth factor for breast cancer cells.

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